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Metabolism of Quaternary Carbon Compounds: 2,2-Dimethylheptane and Tertbutylbenzene

D. CATELANI, A. COLOMBI, C. SORLINI, AND V. TRECCANI*

Institute of Agrarian and Technical Microbiology, University of Milano, 20133 Milan, Italy

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Two *Achromobacter* strains capable of utilizing 2,2-dimethylheptane or tertbutylbenzene as the sole carbon and energy source were isolated from wastewater. Pivalic acid was found in the cultures of *Achromobacter* A_1 containing 2,2-dimethylheptane. From cultures of *Achromobacter* A_2 in the presence of tertbutylbenzene, a diol was isolated and identified as 2,3-dihydro-2,3-dihydroxytertbutylbenzene. Evidence for *meta* cleavage of the aromatic ring and for accumulation of pivalic acid in the cultures was also obtained. A metabolic pathway for tertbutylbenzene is suggested.

Ternary and quaternary carbon compounds are described as molecules strongly resistant to biodegradation (8); in addition, little is known about their metabolic pathways. Only Kerstin (7) reported the isolation of microorganisms capable of growing on 2,2,4-trimethylheptane, and Mohanrao and McKinney (9) showed the degradation of different acids with quaternary C atoms by activated sludges.

In our laboratory, bacteria have been isolated that are able to grow on 2,2-dimethylsuccinic acid; the metabolic pathways for these compounds have been studied (12). These structures are of particular interest since they are involved in many classes of compounds widely used in agriculture (pesticides) and in industrial and domestic detergents (surfactants). Their degradation mechanisms must be fully clarified to minimize the danger of pollution by synthesizing and using only biodegradable compounds. In the present paper, studies on the microbial degradation of two compounds with quaternary C atoms, 2,2-dimethylheptane and tertbutylbenzene, are described.

MATERIALS AND METHODS

Organisms and growth conditions. Two gram-negative rods, A_1 and A_2, tentatively assigned to the genus *Achromobacter*, were isolated from wastewater by elective culture methods in the presence of 2,2-dimethylheptane and tertbutylbenzene, respectively. The isolated strains were maintained on Raymond and Davis (11) mineral salts liquid medium supplemented with a few drops of 2,2-dimethylheptane or tertbutylbenzene.

Although the hydrocarbon substrates were not sterilized, controls without inoculum showed no growth. When required, plates of the same mineral salts medium solidified with Difco agar (1.8%, wt/vol) were inoculated and inverted; a few drops of hydrocarbon substrate were then added to a filter paper dish in the lid. The two strains were frequently plated for reisolation on Difco nutrient agar.

For manometric experiments and large-scale incubations with resting cells, the organisms were cultured on the same mineral salts medium supplemented with a few drops of 2,2-dimethylheptane or tertbutylbenzene and with 0.2% (wt/vol) sodium glutamate. Preliminary experiments showed that glutamate did not repress the synthesis of enzymes involved in benzene ring fission. The cells were harvested after 48 h of incubation, washed twice by centrifugation in 0.02 M potassium phosphate buffer (pH 7.0), and resuspended in the same buffer to give a total N content of 0.3 mg/ml. O_2 uptake and CO_2 formation were determined in a Warburg respirometer at 30°C.

Spectral analyses. Ultraviolet spectra were recorded in a Perkin-Elmer model 124 double-beam spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, Bucks, U.K.); an Infracord model 137 (Perkin-Elmer Ltd.) was used to obtain infrared spectra (in Nujol). Mass spectra were recorded on an LKB model 9000 spectrometer at 70 eV.

Chemical determinations. Catechols were tested by the $FeCl_3$ method (14). Diols were tested by the method of Boyland and Wiltshire (2). Methyl esters were prepared with diazomethane in diethyl ether (14). Picolinate derivatives were prepared in ammonium acetate solution as reported by Canonica et al. (3).

Chromatography. Thin-layer chromatography was done on silica gel plates (Bakerflex IB-F no. 5002; Y. T. Baker Chemical Co., Deventer, Holland) activated by heating at 110°C for 30 min. Preparative thin-layer chromatography was performed with silica gel plates (2 mm thick; E. Merck, Darmstadt, no. 5717) activated in the same way. The developing solvent system was chloroform–hexane–acetic acid (2:8:1; vol/vol/vol); spots were revealed by absorbance with an ultraviolet lamp (at 254 nm). Gas-liquid chromatography of phenolic substances was performed by using a Carlo Erba model G.T. 200 gas chromatograph with a flame ionization detector. A stainless-steel column (2 m by 2 mm ID) packed

with 1% SE30 60 to 80 mesh on silanized Chromosorb G was used at the following temperatures: column, 190°C; injector, 230°C; detector, 220°C. The carrier gas was N_2 at 35 ml/min. For gas-liquid chromatography of short-chain aliphatic acids, the same column was used at the following temperatures: injector, 80°C; column, 70°C; detector, 80°C. Methyl esters of acids were gas chromatographed in a stainless-steel column, of the same dimensions, packed with 1% ethylene glycol succinate (Carlo Erba LAC886) on 60 to 80 mesh silanized Chromosorb G at the following temperatures: injector, 120°C; column, 120°C; detector, 130°C. N_2 was supplied as described above. When the gas chromatograph was combined with the LKB model 9000 mass spectrometer, He was used as carrier gas at 30 ml/min; columns and temperatures were as reported above.

Chemicals. 2,2-Dimethylheptane, 2,2-dimethylhexane, 2,2-dimethylpentane, 3,3-dimethylbutyric acid, and pivalic acid were supplied by Fluka AG, Buchs, Switzerland; tertbutylbenzene was from Eastman Organic Chemicals, Rochester, N.Y.; 2-tertbutylphenol and 3,4-dihydroxytertbutylbenzene were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; 3-tertbutylphenol was from K & K Laboratories Inc., Plainview, N.Y.

Chemical synthesis of 2,3-dihydroxytertbutylbenzene. An 11.0-g amount of catechol was dissolved in 50 ml of CS_2; 3.5 g of anhydrous $FeCl_3$ was added, and the mixture was heated to 50 to 60°C in a water bath. Tertbutylchloride (9 ml) was slowly added while the mixture was stirred vigorously. Then the reaction mixture was refluxed for 1 h, and a slight excess of a aqueous Na_2SO_3 (10%, wt/vol) was added to reduce the $FeCl_3$. The organic layer was then separated and washed with three 5-ml portions of water. After drying with anhydrous $MgSO_4$ and evaporating of the solvent, a crystalline mass was obtained that was mainly unreacted catechol. When the crystals were repeatedly extracted with pentane, evaporation of the collected solvent led to a crystalline mass. Thin-layer chromatography of this residue showed that it was a mixture of three compounds: the first had an R_f of 0.11, the same as catechol; the second had an R_f of 0.21, the same as 3,4-dihydroxytertbutylbenzene; and the third had an R_f of 0.25. Preparative thin-layer chromatography of the crude residue allowed isolation of a substance with an R_f of 0.25 by $CHCl_3$ extraction of the corresponding band. Evaporation of the solvent gave an oily residue. When this was distilled in a conventional microdistillation apparatus, a viscous light brown liquid was obtained, which gave a positive $FeCl_3$ reaction. This substance was unstable so that storage was possible only under N_2 in the cold (−20°C). Its mass spectrum showed an M^+ of 166 and was comparable to, but not superimposable on, that of 3,4-dihydroxytertbutylbenzene; also, its infrared spectrum was different from that of 3,4-dihydroxytertbutylbenzene in the fingerprint region. The chromatographic retention time of the unknown substance was very different from those of catechol and 3,4-dihydroxytertbutylbenzene. These physicochemical properties are in agreement with a compound having the structure of 2,3-dihydroxytertbutylbenzene.

RESULTS

Metabolism of 2,2-dimethylheptane. *Achromobacter* A_1 grew well when 2,2-dimethylheptane, 2,2-dimethylhexane, 2,2-dimethylpentane, 2,2-dimethylmalonate, or isobutyrate was supplied as the only carbon and energy source, but it did not grow well on 3,3-dimethylbutyrate, 2,2-dimethylsuccinate, or pivalate.

Washed suspensions of strain A_1 grown on 2,2-dimethylheptane showed an immediate O_2 uptake in presence of 2,2-dimethylheptane, 2,2-dimethylhexane, and 2,2-dimethylpentane, but not in presence of isobutyrate, 2,2-dimethylmalonate, or 2,2-dimethylsuccinate. Pivalic acid was oxidized after a 10-min lag phase, with an O_2 uptake of 2 mol/mol and the formation of 2 mol of CO_2 per mol of substrate added. In presence of 3,3-dimethylbutyrate, the O_2 uptake was only 1 mol/mol of substrate added, with a 10-min lag phase; no CO_2 was formed.

To isolate sufficient amounts of 2,2-dimethylheptane metabolites for identification, several 750-ml Erlenmeyer flasks containing 100 ml of Raymond and Davis (11) mineral salts medium and a few drops of substrate were inoculated with strain A_1 and incubated at 27°C by shaking. At 4-h intervals, between 0 and 48 h, samples were taken and checked for accumulation of intermediates. For each sample the reaction mixture was made alkaline (pH 10) with aqueous 5% (wt/vol) K_2CO_3 and centrifuged to remove inorganic phosphate precipitate and cells. After paper filtration, three extractions were performed with 30 ml of pentane to eliminate the unmetabolized hydrocarbon; then the aqueous phase was acidified to pH 3 with 6 N HCl and extracted with diethyl ether. The organic phase was cooled to −20°C, the ice that formed was filtered through hydrophilic cotton, and the organic solvent was concentrated at room temperature to a small volume under reduced pressure. The residue was divided into two portions. The first was directly analyzed by gas chromatography-mass spectrometry. The other was treated with a diethyl ether solution of diazomethane and also analyzed by gas chromatography-mass spectrometry. The mass spectra of the two samples showed the presence of pivalic acid and methyl pivalate, respectively.

Experiments with resting cells were carried out essentially in the same manner. Several Erlenmeyer flasks containing 30 ml of a washed suspension of cells (equivalent to a total N content of 0.30 mg/ml) grown in the presence of 2,2-dimethylheptane, 60 ml of phosphate buffer (pH 7.0), and a few drops of 2,2-dimethylheptane as substrate were incubated at 27°C with shaking. At 1-h intervals, samples

were checked as before; only pivalic acid and methyl pivalate were detected.

Metabolism of tertbutylbenzene. *Achromobacter* A_2 grew well on tertbutylbenzene and 2-tertbutylphenol but not on benzoate or pivalate. Washed suspensions of strain A_2 grown on tertbutylbenzene showed an immediate O_2 uptake in presence of tertbutylbenzene and 2,3-dihydroxytertbutylbenzene; this last compound gave rise in the reaction mixture to a yellow compound showing a λ_{max} of 410 nm at pH 12 and of 318 nm at pH 3. By treatment with ammonium acetate (3), this compound was converted to another substance showing an ultraviolet spectrum characteristic of picolinate derivatives (λ_{max} = 275 nm).

No oxygen uptake was observed with 3,4-dihydroxytertbutylbenzene or pivalic acid.

The 24-h cultures of *Achromobacter* A_2 incubated in the presence of tertbutylbenzene showed a weak positive diol test. When the concentration of the diol in 20-liter cultures was at a maximum, the reaction mixture was made pH 9 with aqueous 5% (wt/vol) K_2CO_3. Inorganic phosphate precipitate and cells were removed by centrifugation and paper filtration, and the culture fluid was extracted repeatedly with several 250-ml portions of peroxidase-free diethyl ether. The organic layers were collected and cooled to −20°C to eliminate water. The solvent was then evaporated in the cold under reduced pressure, and the residue was redissolved in a small volume of water. A sample of this solution was checked for the presence of diols, and the reaction was positive. The crude diol was easily converted into a mixture of two phenols by heating in acidic conditions (1). These dehydration products were analyzed by gas chromatography-mass spectrometry and, by comparison with authentic specimens, were shown to be 2-tertbutylphenol and 3-tertbutylphenol.

Many attempts were made to crystallize the diol from the crude residue, or even to purify it, but no positive result was obtained, presumably because of the relative instability of the compound, which was decomposed in a few minutes.

The 48-h cultures of *Achromobacter* A_2 incubated in the presence of tertbutylbenzene were made alkaline (pH 10) with aqueous 5% (wt/vol) K_2CO_3, centrifuged, filtered, extracted with diethyl ether, and analyzed as described for A_1 cultures; only pivalic acid and methyl pivalate were detected.

Similar experiments carried out with resting cells, as reported for strain A_1, in the presence of tertbutylbenzene gave the same results. No evidence was obtained for pivalate metabolism either by cultures or by resting cells under the prevailing experimental conditions.

DISCUSSION

Pivalic acid was found to be a metabolite of both 2,2-dimethylheptane and tertbutylbenzene. Growth experiments showed that neither isolated strain is able to utilize pivalic acid as the sole carbon and energy source, even though resting cells of *Achromobacter* A_1 are able to oxidize pivalic acid with an O_2 uptake of 2 mol/mol and CO_2 release of 2 mol/mol of substrate added. At the present time, our attempts to isolate microorganisms able to grow on pivalic acid have been unsuccessful.

The first detectable metabolic step in tertbutylbenzene degradation by *Achromobacter* A_2 is the formation of a diol. The identification of 2- and 3-tertbutylphenols as decomposition products in acidic conditions of the diol allowed us to suggest the structure of 2,3-dihydro-2,3-dihydroxytertbutylbenzene. In addition, Warburg experiments showed that the same strain grown on tertbutylbenzene was unable to oxidize 3,4-dihydroxytertbutylbenzene, whereas 2,3-dihydroxytertbutylbenzene was rapidly oxidized, forming a yellow compound whose chemical and physicochemical properties were consistent with a structure related to α-hydroxymuconic semialdehyde. This suggests that the ring fission of 2,3-dihydroxytertbutylbenzene proceeds via *meta* cleavage. No metabolic product other than pivalic acid was found in the cultures. From these results the metabolic pathway shown in Fig. 1 for tertbutylbenzene

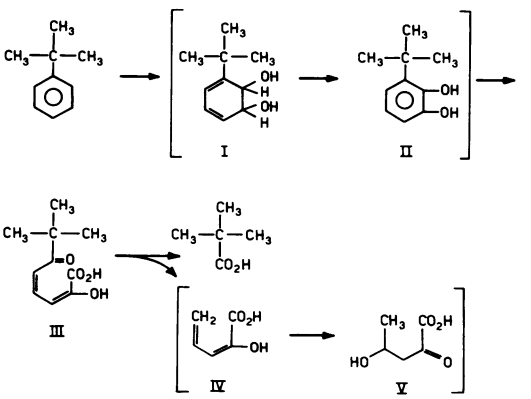

FIG. 1. *Proposed pathway of tertbutylbenzene by Achromobacter. (I) 2,3-Dihydro-2,3-dihydroxytertbutylbenzene; (II) 2,3-dihydroxytertbutylbenzene; (III) 2-hydroxy-6-oxo-7,7-dimethylocta-2,4-dienoic acid; (IV) 2-hydroxypenta-2,4-dienoic acid; (V) 4-hydroxy-2-oxovaleric acid. Square backets denote that the compounds have not been isolated and identified.*

is suggested. This pathway, involving an extradiol vicinal cleavage, in the nomenclature reported by Nozaki et al. (10), is identical to those proposed for other aromatic compounds (13), such as phenylpropionic acid (6), 3- and 4-methylcatechol (3, 5), 3-phenylpentane (1), and biphenyl (4).

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